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(54) Title: METHOD OF COMBATING VIRAL INFECTIONS

(57) Abstract

A method for combating viral infection in a human or animal patient, comprises administering to the patient histocompatible blood cells or bone marrow stem cells which have been transformed with a nucleotide sequence coding for a protein or polypeptide which, on expression by the cells will reduce the rate at which the superinfecting virus particles can enter the cells. The nucleotide sequence may be a sequence or gene coding for all or an immunologically-active portion of a HIV envelope protein such as gp160, gp120 or gp41.

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METHOD OF COMBATING VIRAL INFECTIONS

This invention relates to a method of combating viral infections, and in particular it relates to a method of combating infections with retroviruses.

BACKGROUND

Retroviruses are RNA viruses that can make a DNA copy of their genome using a virus-encoded enzyme called reverse transcriptase. The DNA copy (ies) can become randomly integrated into the genome of eukaryotic cells. These integrated viral genomes may remain silent, may produce infectious virus, or may be oncogenic. Rarely, infection with a retrovirus is lytic for the infected cell. Large numbers of retroviruses have been isolated from mice and many have no obvious pathologic effect. However because retroviruses contain onc genes, which may be derived from cellular analogues that control cell division, they have been extensively studied in an attempt to understand oncogenesis. For many years the relevance of such work to man was obscure but the discovery of three disease-causing retroviruses in man has thrust retroviral research to the forefront of medical research.

Retroviruses that affect man include the human T-lymphotropic viruses HTLVI and HLTVII, which can cause leukaemias, and the human immunodeficiency virus (HIV or HTLVIII) which causes the acquired immunodeficiency syndrome (AIDS). The AIDS virus is somewhat unique in that it causes a lytic infection of lymphocytes, thus inducing a severe and chronic suppression of the immune response of the host, ultimately resulting in a complete collapse of the body's defences against infection and the like. AIDS sufferers normally die as the result of uncontrolled secondary infections due to viruses, fungi, protozoa and bacteria, or as a result of uncontrolled cancers or the like. The total number of retrovirus-infected individuals in the western world may be 10 million and is in any event increasing. There is at present no cure for AIDS, and the disease seems to be uniformly fatal although the virus can take many years after initial infection to cause disease. World-wide efforts to date to produce a vaccine against AIDS by traditional methods have not been effective, and in the absence of an effective vaccine the main defences against the disease have relied on education directed to prevention of the spread of the virus throughout the community.

Retroviruses are divided into groups based on their ability to interfere with infection of their host cell by other retroviruses. When a cell is infected with a virus in a particular interference group, no other virus in that group can subsequently infect the cell, that is the cell cannot be subsequently "superinfected". Infection of the cell by viruses in other interference groups is unaffected. This phenomenon of interference is believed to be most likely due to saturation of available virus-binding sites on the cell surface by viral envelope

components made within the infected cell. However, genetic activation of other regulatory mechanisms cannot be excluded.

The mechanism of viral interference was elucidated in 1966 by Steck and Rubin (1,2) who showed that viral interference reduces the rate at which superinfecting virus particles penetrate the cell. Cell surface receptors specific for the envelope of the virus being produced are occupied by virus envelope molecules synthesised within the virus-producing cell, and are thus not available for interaction with superinfecting virus particles.

The AIDS virus, HIV, includes major proteins in its outer envelope which are glycoproteins known as gp120 and gp41. The virus initially produces a large envelope protein, gp160, before splitting it into the two smaller proteins. HIV infects cells primarily because the glycoprotein gp120 which is positioned on the envelope of the virus and on the membrane of cells infected with HIV, binds to a cell surface determinant called CD4, which is found predominantly on helper type thymus-derived lymphocytes (3,4). $CD4^+$ thymus-derived lymphocytes, are the primary target of HIV, and infection and subsequent loss of these cells results in immunosuppression and the catastrophic adventitius sequelae that are characteristic of HIV infection.

As previously described, a major defect in AIDS is loss of immune function, particularly T-cell mediated immune function as a result of lymphocyte destruction by the AIDS virus. One means for reconstituting immune function would be by transplantation of histocompatible blood cells or bone marrow stem cells, however in the HIV infected patient these transplanted cells would of course themselves be infected and destroyed.

It is one object of the present invention to provide a method whereby such cells can be transplanted without resultant destruction by the virus.

SUMMARY OF THE INVENTION

The present invention provides a method for combating viral infection in a human or animal patient, which comprises administering to the patient histocompatible blood cells or bone marrow stem cells which have been transformed with a nucleotide sequence coding for a protein or polypeptide which, on expression by the cells, will reduce the rate at which superinfecting virus particles can enter the cells.

In another aspect, the present invention provides a therapeutic or prophylactic composition for combating viral infection in a human or animal patient, which comprises histocompatible blood cells or bone marrow stem cells which have been transformed with a nucleotide sequence coding for a protein or polypeptide which, on expression by the cells, will reduce the rate at which superinfecting virus particles can enter the cells.

As previously described, the present invention has particular application in combating retroviral infection, most particularly HIV infection, and in one particularly preferred embodiment of the present invention the histocompatible blood cells or bone marrow stem cells are transformed with a nucleotide sequence or gene coding for all or an immunologically-active portion of a HIV envelope protein. Most preferably, the cells are transformed with a nucleotide sequence or gene coding for all or an immunologically-active portion of the HIV envelope proteins gp160, gp120 or gp41, or any portion of the gp160 gene encoding an "interfering" polypeptide.

Further, selected blood cells expressing HIV envelope proteins may be useful as potent immunogens inducing strong cell mediated immunity to HIV.

In recent years, a number of retroviral vectors have become well known (5a, 5b, 6) and available. These vectors can enter cells and express the genes or nucleotide sequences they carry without producing infectious virus. Such vectors include, for example, non-replicating viral genomes which do not contain the gene for reverse transcriptase and so cannot reproduce, or modified viral genomes which do not contain genes for structural components of the virus envelope and so cannot make infectious virus. In the performance of the present invention, for example in the production of cells transformed with HIV gp160, gp120 or gp41 nucleotide sequences, retroviral vectors are used to construct artificial HIV-like viral genomes. These constructs, of course, do not have the pathogenicity of HIV since they cannot replicate in the transformed cells, however they do contain the gene or similar sequence for gp160, gp120 or gp41 under control of either a modified retroviral promoter or some other promoter.

The transformed cells may be used in accordance with the present invention for combating appropriate viral infections. Thus, they may be used therapeutically in the treatment of infected patients or they may be used prophylactically in order to prevent or minimise infection by the virus.

In one preferred embodiment of the invention, bone marrow stem cells are transformed with a retroviral vector which carries the gene coding for the envelope glycoprotein gp160 or gp120 from HIV. This gene enables the transformed bone marrow cells to express gp160 or gp120 which will bind to the cell surface determinant CD4

of helper type thymus-derived lymphocytes. The surface receptors of these cells will thus become saturated with gp120, and not be available for interaction with super-infecting HIV particles.

The methods used in this aspect of the invention, include construction of a viral vector containing the gene for gp160 downstream of a suitable promoter, and the use of this vector to transfect bone marrow stem cells and lymphocytes in vitro. The cells expressing gp160 or gp120 and gp41 can then be exposed to HIV, and examined for production of infectious virus and/or reverse transcriptase (an indicator of retroviral infection).

An alternative, and safer method, for testing cells that express HIV gp160 or gp120 for resistance to superinfection would be to use radiolabelled, purified gp120 antigen to measure available gp120 binding sites. Cells that already produce gp120, and have therefore saturated their gp120 binding sites, will not bind significant amounts of the labelled ligand.

As previously described, the present invention particularly relates to combating retroviral infections, and in addition to its use in relation to HIV or HLTVIII described in detail, the invention may be used in therapeutic or prophylactic treatment of leukaemias resulting from HTLVI or HLTVII infections in humans. In the treatment of such leukaemias, known chemotherapy and/or radiotherapy procedures are used to destroy the leukaemic cells, and the patient may then be treated by administration of appropriately transformed cells in accordance with the present invention.

The present invention is further illustrated in one embodiment by the following Example which relates to the construction of cells transformed with the HIV gp160 gene.

EXAMPLE

In this Example, standard techniques were used as described for example, in Maniatis "Molecular Cloning, A Laboratory Manual". Restriction enzymes were used in accordance with manufacturer's directions.

a. Expression of HIV-1 envelope glycoprotein gp160 in a retroviral shuttle vector.

The retroviral shuttle vector, fpGV-1, (5a, 5b) was used to express the HIV-1 envelope glycoprotein gp160. Vector fpGV-1 was derived from the HT-1 strain of Moloney Sarcoma Virus (MSV), and contains the bacterial ColE1 origin of DNA replication, the neomycin resistance gene from the transposon Tn5 and the long terminal repeats (LTRs) of HT-1 MSV. It does not contain any sequences related to the transforming mos gene (Figure 1). The HIV-1 envelope glycoprotein gp160 gene was excised from the commercially available plasmid pBH10 (Biotech Research Labs., U.S.A.) which contains the HIV-1 9kb partial genome from Sst1 to Sst1 sites (Figure 2). The strategy of cloning of the gp160 gene into the retroviral shuttle vector fpGV-1 is shown in Figure 3. The pBH10 plasmid DNA was digested with restriction endonuclease Xho1 and the sticky ends of the DNA were blunt-ended using T4 DNA polymerase. The DNA was then digested with restriction endonuclease Sal1 and separated on a 1% low melting point agarose gel. A 3.1kb DNA fragment was isolated from the gel using hot phenol extraction method. Vector fpGV-1 plasmid DNA was digested with restriction endonuclease EcoR1 and the sticky ends of the DNA were blunt-ended using T4 DNA polymerase. The vector DNA was then digested with restriction endonuclease Sal1. The linearised vector DNA was ligated with the 3.1kb Xho1 (blunt-ended)-Sal1 DNA fragment isolated from pBH10 and transformed into E.coli DH5α. A clone containing the 3.1kb fragment was further

analysed with restriction endonuclease digestion : Sal1+BamH1 (6.2kb and 2.7kb); Hind3 (5.3kb, 2.2kb and 1.4kb). This clone was named as HIV-F1 contains the HIV-1 envelope glycoprotein gp160 gene which is orientated in the same transcription direction as the SV40 early promoter (shown in Figure 3).

b. Production of $CD4^{\pm}$ cell lines.

Peripheral blood mononuclear cells (10^6/ml) were stimulated with concanavalin A (10 µg/ml) for 4 days. Activated T cells were washed, subcultured at $3x10^5$/ml in tissue culture medium containing optimum growth concentrations of interleukin-2 (IL-2). The activated cells were stored in liquid N_2. As required, cells were thawed and treated with OKT8 for 30 min on ice followed by rabbit complement for 1 hr at 37°C. Treated cells were washed and cultured in round well trays in IL-2 at $2x10^4$/well in 200µl. Growth of the activated T cells was inactivated by periodic addition of IL-2 and by stimulation with 5 µg/ml of phytohaemagglutinin and a 0.05% suspension of sheep red blood cells. Activated T cells were monitored by FACs analysis for T cell surface markers. Populations generated were 90-95% $CD4^+$, 0% $CD8^+$, with remaining cells being $CD16^+$ NK cells.

c. Transfection of COS-1 and $CD4^+$ lymphocytes.

COS-1 cells were seeded at $5x10^5$ per 60mm diameter petri dish, grown overnight in Dulbecco's modified Eagle's medium (DMEM) (Flow Labs, Sydney, Australia), supplemented with 10% fetal calf serum (FCS), then transfected with HIV-F1 (20 µg DNA per 10^6 cells) by calcium phosphate precipitation and glycerol shock (15% glycerol for three minutes). The cells were then washed in DMEM containing 10% FCS, rested in 5ml of the same medium and incubated at 37°C in 5% CO_2.

$CD4^+$ lymphocytes produced as described above were transfected by allowing them to settle with the $CaPO_4$ precipitate, decanting off supernatant, then applying glycerol shock as above. Cells were resuspended in growth medium and plated in 96 well round bottom plates at $2x10^4$ cells/well.

Cells were fed every three days. After the first 72 hours G418 antibiotic was added to test and control cultures. Nontransfected $CD4^+$ cells died within 72 hours and COS-1 cells within 96 hours. Cells transfected with HIV-F1 and selected by their resistance to G418 were tested in a fluorescence antibody test using anti-HIV serum from an AIDS patient. These cells were found to be 100% positive, confirming expression of gp160 by these cells.

Similar methods to those described above have also been used to transfect human bone marrow cells.

As an alternative to the calcium phosphate precipitation/glycerol shock technique, transfection of large numbers of bone marrow cells or lymphocytes can be most effectively achieved using the electroporation technique. In this technique, cells suspended in buffer containing positive retroviral vector are exposed to an electric pulse. This causes temporary pore formation in the cell membrane allowing the vector to enter the cell. Transplant cells transfected in this way and containing, for example, the gene or part of the gene for gp160, could be injected directly back into the patient without prolonged culture.

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1. Steck, F.T. and Rubin, H. (1966a). Virology 29: 628-641.

2. Steck, F.T. and Rubin (1966b). Virology 29: 642-653.

3. Mathews, T.J. et.al. Proc.Natl.Acad.Sci.(USA) 84: 5424-5428.

4. Dalgleish, A.G. et.al. (1987). Nature 312: 763-766.

5a. Jhappan, C. et.al. (1986) J.Virology 60: 750-753.

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6. Hapel, A.J. et.al. (1986) Lymphokine Research 5: 249-254.

CLAIMS:

1. A method for combating viral infection in a human or animal patient, which comprises administering to the patient histocompatible blood cells or bone marrow stem cells which have been transformed with a nucleotide sequence coding for a protein or polypeptide which, on expression by the cells will reduce the rate at which superinfecting virus particles can enter the cells.

2. A method according to claim 1 for combating infection with the human immunodeficiency virus (HIV), wherein said histocompatible blood cells or bone marrow stem cells have been transformed with a nucleotide sequence or gene coding for all or an immunologically-active portion of a HIV envelope protein.

3. A method according to claim 2, wherein said cells have been transformed with a nucleotide sequence or gene coding for all or an immunologically-active portion of the HIV envelope proteins gp160, gp120 or gp41.

4. A therapeutic or prophylactic composition for combating viral infection in a human or animal patient, which comprises histocompatible blood cells or bone marrow stem cells which have been transformed with a nucleotide sequence coding for a protein or polypeptide which, on expression by the cells, will reduce the rate at which superinfecting virus particles can enter the cells.

5. A composition according to claim 4, for combating infection with the human immunodeficiency virus (HIV), wherein said histocompatible blood cells or bone marrow stem cells have been transformed with a nucleotide sequence or gene coding for all or an immunologically-active portion of a HIV envelope protein.

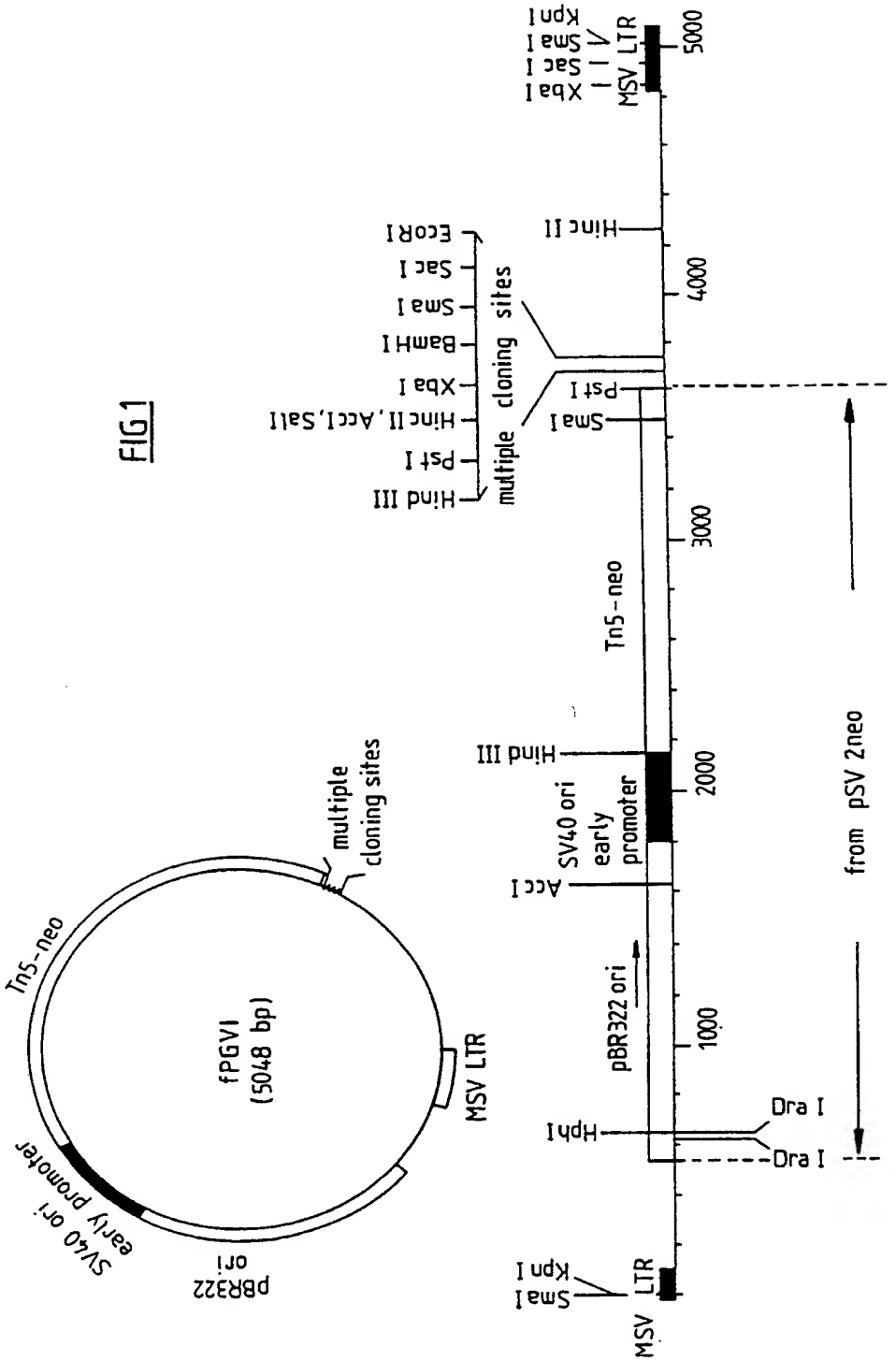
6. A composition according to claim 5, wherein said cells have been transformed with a nucleotide sequence coding for all or an immunologically-active portion of the HIV envelope proteins gp160, gp120 or gp41.

7. A method for the preparation of a therapeutic or prophylactic composition according to claim 4, which comprises the steps of preparing a viral vector containing said nucleotide sequence under operative control of a suitable promoter sequence, and then transfecting said cells with said viral vector.

8. A method according to claim 7, wherein said viral vector is a retroviral vector.

9. A method according to claim 8, wherein said retroviral vector contains the nucleotide sequence or gene coding for the HIV envelope proteins gp160, gp120 or gp41 downstream of a suitable promoter sequence.

FIG 1


fPGVI
(5048 bp)
multiple cloning sites
Tn5-neo
MSV LTR
pBR322 ori
SV40 ori early promoter
Hind III
Pst I
Hinc II, Acc I, Sal I
Xba I
BamH I
Sma I
Sac I
EcoR I
multiple cloning sites
Hinc II
Xba I
Sac I
Sma I
Kpn I
MSV LTR
Sma I
Kpn I
Acc I
Hind III
Hph I
Dra I
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SV40 ori early promoter
Tn5-neo
from pSV 2neo
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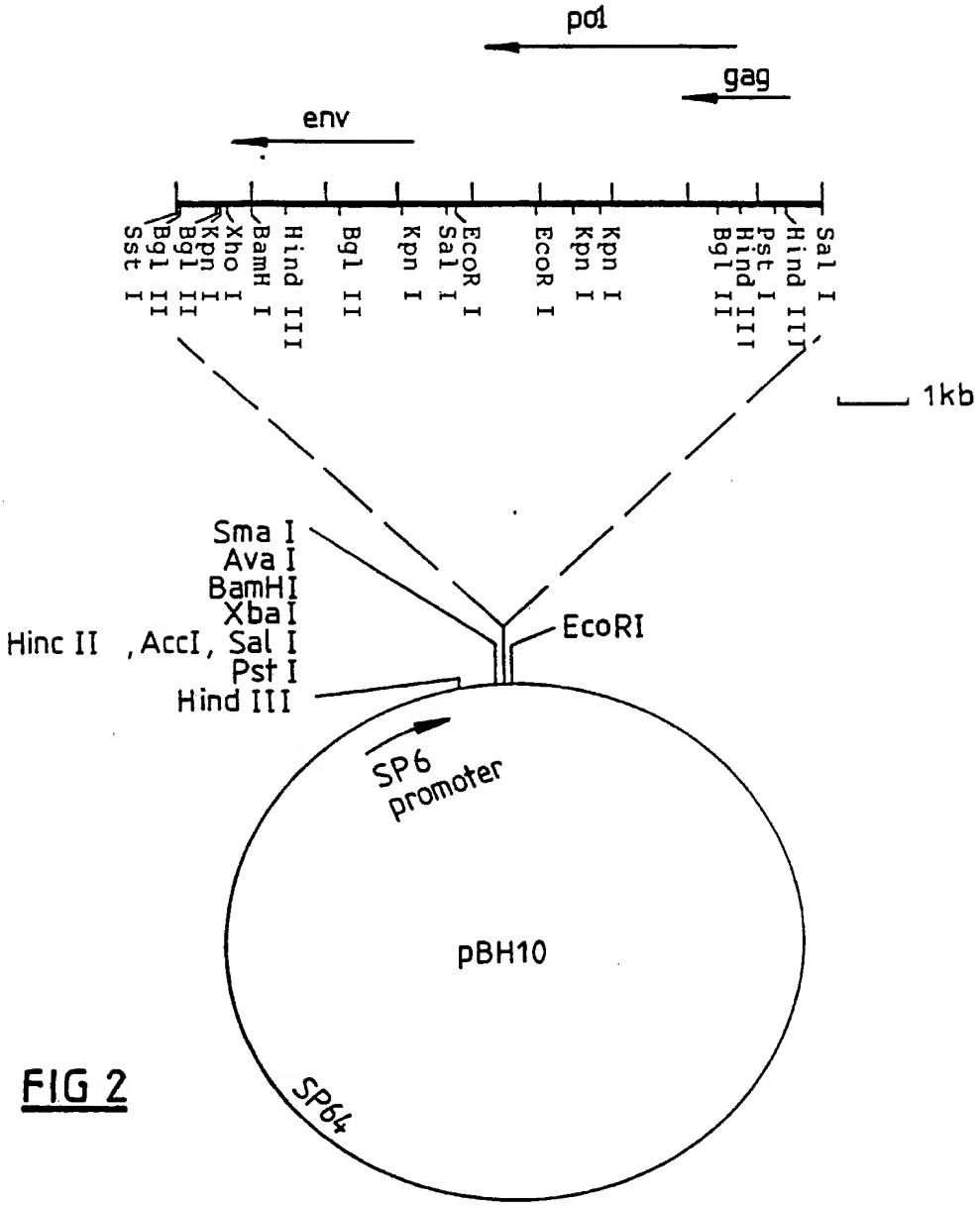
2000
3000
4000
5000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gag
env
Sst I
Bgl II
Bgl II
Kpn I
Xho I
BamH I
Hind III
Bgl II
Kpn I
EcoR I
Sal I
EcoR I
Kpn I
Kpn I
Bgl II
Pst I
Hind III
Hind III
Sal I
1kb
Sma I
Ava I
BamHI
Xba I
Hinc II , AccI, Sal I
Pst I
Hind III
EcoRI
SP6
promoter
pBH10
SP64

FIG 2

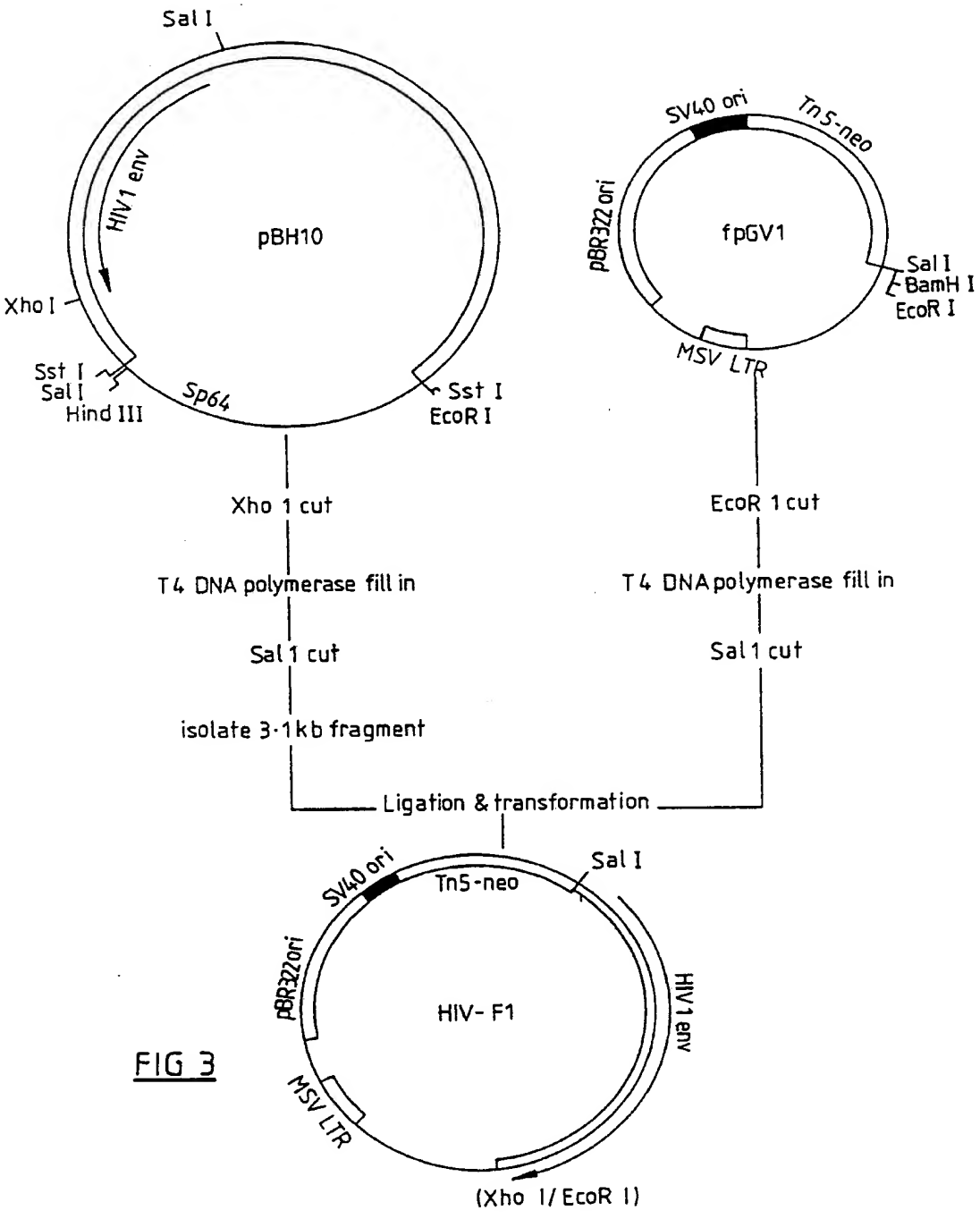
Sal I
HIV 1 env
pBH10
Xho I
Sst I
Sal I
Hind III
Sp64
Sst I
EcoR I
SV40 ori
Tn5-neo
pBR322 ori
fpGV1
Sal I
BamH I
EcoR I
MSV LTR
Xho 1 cut
EcoR 1 cut
T4 DNA polymerase fill in
T4 DNA polymerase fill in
Sal 1 cut
Sal 1 cut
isolate 3·1 kb fragment
Ligation & transformation
SV40 ori
Tn5-neo
Sal I
pBR322ori
HIV- F1
HIV 1 env
MSV LTR
(Xho I/ EcoR I)

FIG 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 88/00474

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 15/00, 5/00, A61K 35/18, 35/14

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA : Keywords : HIV, Human Immunodeficiency Virus, HTLV, Human T-cell Lymphotropic Virus, LAV, Lymphadenopathy Associated Virus, ARV, AIDS-related Virus, AIDS, Acquired Immune Deficiency Syndrome, Env and Protein

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : C12N 15/00, 5/00, 5/02, A61K 35/14, 35/18
Chemical Abstracts : Keywords as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	AU,A, 23800/88 (UPJOHN COMPANY) 10 October 1988 (10.10.88)	(4,7)
A	AU,A, 62992/86 (S. HU) 9 April 1987 (09.04.87)	
A	Kieny, M.P., et al, Biotechnology, Volume 4, issued September 1986, "AIDS virus env protein expressed from a recombinant vaccinia virus", see pages 790-795.	
A	Chakrabarti, S., et al, Nature, Volume 320, issued 10 April 1986 (London), "Expression of the HTLV-III envelope gene by a recombinant vaccinia virus", see pages 535-537.	

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 February 1989 (20.02.89)	2 MARCH 1989 (02.03.89)
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	R. DAL BON

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 1-3, because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1 (iv): method of treatment of the human or animal body by surgery or therapy.

2. [ ] Claim numbers....., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 88/00474

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members
AU 23800/88	WO 8807080
AU 62992/86	BE 905492, CN 86106632, DK 4554/86, FI 863848, FR 2593519, GB 2181435, HU 42133, IL 80073, JP 63068075, LU 86608, NL 8602422, NO 863803, PT 83434, SE 8604007, WO 8702038, ZA 8607281

END OF ANNEX